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Comparative Genomic Analysis of *Campylobacter jejuni* Strains Reveals Diversity Due to Genomic Elements Similar to Those Present in *C. jejuni* Strain RM1221[▽]

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Analysis of the complete genomic sequence of *Campylobacter jejuni* strain RM1221 identified four large genomic elements, *Campylobacter jejuni-i*ntegrated *e*lements (CJIEs), that were absent from *C. jejuni* strain NCTC 11168. To further investigate the genomic diversity of *Campylobacter*, we conducted a comparative genomic analysis from a collection of 67 *C. jejuni* and 12 *Campylobacter coli* strains isolated from various geographical locations and clinical and veterinary sources. Utilizing PCR, we demonstrated that 55% of the *C. jejuni* strains examined were positive for at least one RM1221-like genomic element and 27% were positive for two or more of these CJIEs. Furthermore, many *C. coli* strains were positive for either genomic element CJIE1 or CJIE3. To simultaneously assess for the presence or absence of several genes that comprise the various CJIEs, we developed a multistrain *C. jejuni* DNA microarray that contained most of the putative coding sequences for strains NCTC 11168 and RM1221. A comparative genomic hybridization (CGH) analysis of 35 of the 67 *C. jejuni* strains confirmed the presence of genomic elements similar to those in strain RM1221. Interestingly, the DNA microarray analysis demonstrated that these genomic elements in the other *C. jejuni* strains often exhibited modular patterns with some regions of the CJIEs present and other regions either absent or highly divergent compared to strain RM1221. Our CGH method also identified 18 other intraspecies hypervariable regions, such as the capsule and lipooligosaccharide biosynthesis regions. Thus, the inclusion of genes from these integrated genomic elements and the genes from the other intraspecies hypervariable regions contributes to a better assessment of the diversity in *C. jejuni* and may increase the usefulness of DNA microarrays as an epidemiological genotyping tool. Finally, we also showed that in CJIE1, a *Campylobacter* Mu-like phage, is located differentially in other strains of *C. jejuni*, suggesting that it may integrate essentially randomly.

Campylobacter jejuni colonizes the intestinal mucosa of most food-producing animals. In cattle, swine, and poultry, *C. jejuni* is a common part of the gastrointestinal microflora (13). Yet, in humans, *C. jejuni* is associated with acute gastroenteritis and is the major cause of bacterial food poisoning worldwide. The majority of *C. jejuni* infections result in uncomplicated gastroenteritis, but the development of the peripheral neuropathies Guillain-Barré and Miller-Fisher syndromes are associated often with prior *C. jejuni* infection (16).

The complete genomic sequence of the *C. jejuni* strain NCTC 11168 established an origin for understanding *C. jejuni* genetic differences that would facilitate identification of those determinants that might contribute to the *C. jejuni* pathogenesis. Using *C. jejuni* strain NCTC 11168 microarrays, several groups have indexed the complete gene contents of several *C. jejuni* strains in relation to strain NCTC 11168 (3, 11, 12, 20, 23). These gene indexing studies identified regions of variability between strain NCTC 11168 and other *C. jejuni* strains, such as the lipooligosaccharide biosynthesis (LOS), capsular biosynthesis (CAP), flagellar modification (FM), and DNA restriction/modification (R/M) loci. Furthermore, DNA sequencing of these strain-specific loci identified variability in both the DNA sequence of common genes and gene composition at each locus (6, 10, 14, 18). Indeed, the complete genomic sequence of a second strain of *C. jejuni*, RM1221, illustrated the breadth of intraspecies genome diversity (4). The genome of RM1221 is syntenic with the genome of *C. jejuni* NCTC 11168 but is disrupted by four genomic islands and smaller gene clusters (4). The four genomic islands in strain RM1221 are referred to as *Campylobacter jejuni-i*ntegrated *e*lements (CJIEs). The first genomic island, CJIE1, located upstream of *argC* (CJE0275), is a *Campylobacter* Mu-like phage (also termed CMLP1) encoding several proteins with similarity to bacteriophage Mu and other Mu-like prophage proteins (15). CJIE2 and CJIE4 have several genes predicted to encode phage-related endonucleases, methylases, or repressors and are integrated into the 3′ end of arginyl- and methionyl-tRNA genes, respectively. Finally, CJIE3 (integrated into the 3′ end of an arginyl-tRNA) may be an integrated plasmid. This is based on the observation that 73% (45/62) of the CJIE3 predicted proteins show sequence similarity to those encoded on the *Campylobacter coli* RM2228 megaplasmid (4) or other *Campylobacter* plasmids (2, 17). Also of note, 23% (14/62) of the putative CJIE3 products are similar to proteins found within the 71-kb pathogenicity island of *Helicobacter hepaticus* (HHGI1) (4).

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These findings suggest that these unique integrated elements in strain RM1221 may contribute to an additional level of diversity in *C. jejuni*.

In this study, a comparative genomic analysis was conducted from a collection of 67 *C. jejuni* and 12 *C. coli* strains that were obtained from various geographical locations and clinical and veterinary sources. By using a PCR-based assay and DNA microarrays, we demonstrated that the four CJIEs identified in strain RM1221 were present also in other *Campylobacter* strains that were examined in the present study. The gene indexing analysis performed by using DNA microarrays revealed that several of the coding sequences within these four CJIEs in 26 *C. jejuni* strains were absent or highly divergent compared to strain RM1221, demonstrating an even greater degree of variability within these integrated elements. Furthermore, we observed that, in several *C. jejuni* strains possessing the Mu-like CJIE1, CJIE1 was located at sites distinct from its locations within strain RM1221. Together, these data provide greater insights into the degree of genomic diversity and suggest additional genomic regions to be utilized in differentiating *C. jejuni* strains.

MATERIALS AND METHODS

Bacterial strains and growth. The *Campylobacter jejuni* strains used in this study are shown in Table 1. Strains were grown at 42°C under microaerophilic conditions (8% CO_2, 8% H_2, 4% O_2, 80% N_2) on *Brucella* agar plates or in Mueller-Hinton broth supplemented with 0.025% (wt/vol) $FeSO_4 \cdot 7H_2O$, 0.025% (wt/vol) sodium metabisulfite (anhydrous), and 0.025% (wt/vol) sodium pyruvate (anhydrous).

Element detection. Integrated elements CJIE1, CJIE2, CJIE3, and CJIE4 were detected in *Campylobacter* strains using the following primer sets: CJIE1, 498-1 (5′-GGG ATT AAT AAA AGC TAT ATG-3′) and 498-2 (5′-CAT CTG CAA ATT CAC AAG-3′); CJIE2, 838-1 (5′-CGT AGG AGA ACC AAA AG-3′) and 838-2 (5′-TTC CAT ACC ATT GCA TAA G-3′); CJIE3, 1233-1 (5′-GTA TCA TTT GTT GCT TTG GC-3′) and 1233-2 (5′-TTG AGA GCA TTA ACT AGC-3′); CJIE4, 1617-1 (5′-CAG AGC TTA GAG AAA TCG-3′) and 1617-2 (5′-GAT ATA ATC TCC CCA CC-3′). The expected amplification product sizes are 790 bp for CJIE1, 1,109 bp for CJIE2, 644 bp for CJIE3, and 953 bp for CJIE4.

To determine whether the integrated elements detected in other *Campylobacter* strains were in the same chromosomal location relative to those present in RM1221, genomic DNA from these strains was amplified with primer sets flanking CJIE1 and CJIE3. The flanking primer sets are CJ432 (5′-ATG GGG TAC GAG TGA TGA GTA TAA-3′) and CJ504 (5′-TCT TTT CAA ATT CAA AAC TAA AGC-3′) for CJIE1 and CJ1230-2 (5′-AAG ATA GCC CTT ATG GTA CAC TTT AT-3′) and CJ1299-2 (5′-TTA GGC GTG ATT GCT TAT CTT ATT-3′) for CJIE3. The expected amplification product sizes for NCTC 11168 (negative control) are 581 bp for CJIE1 and 681 bp for CJIE3. The genomic DNA of strains containing elements at these positions would not be expected to amplify under standard PCR conditions.

PCR reagents were supplied by Epicenter (Madison, WI). PCR oligonucleotides were purchased from Operon (Alameda, CA). Each amplification mixture contained 50 ng genomic DNA, 1× MasterAmp *Taq* PCR buffer, 1× MasterAmp *Taq* enhancer, 2.5 mM $MgCl_2$, 2.5 mM concentrations of each deoxynucleoside triphosphate (dNTP), 50 pmol of each primer, and 1 U MasterAmp *Taq* DNA polymerase. PCR was performed using a Tetrad thermal cycler (Bio-Rad, Hercules, CA) with the following amplification parameters: 30 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C and a final extension at 72°C for 5 min. All PCR samples were analyzed by gel electrophoresis, and positive samples were identified based on the presence of bands of the appropriate size.

Inverse PCR. Genomic DNA (1 μg) was digested to completion with HindIII, and 50 ng of digested genomic DNA was then self-ligated overnight. The primers used for each reaction were mu_inv1a (5′-TTA GCA AAA ATC CCA AAT AAT AG-3′) and mu_inv1b (5′-AAT TAT TTA GGG ATA GTT ACA TG-3′). Inverse PCRs were performed on an ABI 9700 thermocycler with the following settings: 1 min at 94°C, 2 min at 50°C, 3 min at 72°C (30 cycles). Each amplification mixture contained 2.5 ng genomic DNA, 1× MasterAmp PCR buffer (Epicenter), 1× MasterAmp PCR enhancer (Epicenter), 2.5 mM $MgCl_2$, 250 μM concentrations of each dNTP, 50 pmol each primer, and 1 U polymerase (Epicenter). Flanking sequences were obtained by sequencing each amplicon using the mu_inv1b primer. The insertion point for each CMLP1 phage was determined by BLASTN analysis against *C. jejuni* (strains RM1221 and NCTC 11168) sequences.

Construction of the *C. jejuni* DNA microarray. DNA fragments of individual open reading frames (ORFs) were amplified using the Sigma-Genosys (The Woodlands, TX) *C. jejuni* ORFmer set specific for strain NCTC 11168 coding sequences and primers from Operon Technologies (Alameda, CA) and designed with ArrayDesigner 2.0 (Premier Biosoft, Palo Alto, CA) specific for unique strain RM1221 coding sequences. Each PCR mixture (total reaction volume, 100 μl) consisted of 1× MasterAmp *Taq* PCR buffer, 1× MasterAmp *Taq* Enhancer, 2.5 mM $MgCl_2$, 200 μM concentrations of each dNTP, forward and reverse primers at 0.2 μM each, 0.5 U of MasterAmp *Taq* DNA polymerase (Epicenter), and approximately 50 ng of genomic DNA (either NCTC 11168 or RM1221). Thermal cycling was performed using a Tetrad thermal cycler (Bio-Rad, Hercules, CA) with the following amplification parameters: 30 cycles of 25 s at 94°C, 25 s at 52°C, and 2 min at 72°C and a final extension at 72°C for 5 min. PCR products were analyzed by gel electrophoresis in a 1% (wt/vol) agarose gel (containing 0.5 μg of ethidium bromide ml^{-1}) in 1× Tris-acetate-EDTA buffer. DNA bands were examined under UV illumination. We successfully amplified a total of 1,530 and 227 PCR products from strains NCTC 11168 and RM1221, respectively. These PCR products were purified on a QIAGEN 8000 robot using a Qiaquick 96-well Biorobot kit (QIAGEN, Valencia, CA), dried, and resuspended to an average concentration of 0.1 to 0.2 μg μl^{-1} in 20 μl of 50% dimethyl sulfoxide containing 0.3× saline-sodium citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). All of the PCR probes were then spotted in duplicate on GAPSII slides (Corning) using an OmniGrid Accent (GeneMachines, Ann Arbor, MI), producing a final array that contained a total of 3,514 features.

Preparation and fluorescent labeling of genomic DNA. Genomic DNA from *C. jejuni* was isolated as described previously (18) or purified using the QIAGEN Dneasy kit (QIAGEN) according to the manufacturer's specifications. For each microarray hybridization reaction mixture, genomic DNAs from the reference strains (an equal amount of DNA from *C. jejuni* strain NCTC 11168 and *C. jejuni* strain RM1221) and a test strain were fluorescently labeled with indodicarbocyanine (Cy5)-dUTP and indocarbocyanine (Cy3)-dUTP, respectively. An aliquot (2 μg) of DNA was mixed with 5 μl 10× NEBlot labeling buffer containing random sequence octamer oligonucleotides (NEB, Beverly, MA.) and water to a final volume of 41 μl. This mixture was heated to 95°C for 5 min and then cooled for 5 min on at 4°C. After this time, the remainder of the labeling reaction components were added: 5 μl of 10× dNTP labeling mix (1.2 mM [each] dATP, dGTP, and dCTP; 0.5 mM dTTP in 10 mM Tris, pH 8.0; 1 mM EDTA), 3 μl of Cy3 dUTP or Cy5 dUTP (GE Biosciences, Piscataway, NJ), and 1 μl of Klenow fragment. The labeling reaction mixtures were incubated overnight at 37°C. Labeled DNA was purified from unincorporated label using Qiaquick PCR cleanup kits and dried by vacuum.

Microarray hybridization. Labeled reference and test DNAs were combined in 45 μl Pronto! cDNA hybridization solution (Corning, Corning, NY) and heated to 95°C for 5 min. Then, 15 μl of the hybridization mixture was put onto a microarray slide and sealed with a coverslip. The microarray slide was placed in a hybridization chamber (Corning) and incubated at 42°C for 18 h. Following hybridization, the slides were washed twice in 2× SSC, 0.1% sodium dodecyl sulfate at 42°C for 10 min, followed by twice in 1× SSC at room temperature for 10 min, and finally twice in 0.2× SSC at room temperature for 5 min. The microarray slides were dried by centrifugation at 300 × *g* for 10 min before scanning. At least two hybridization reactions were performed for each test strain.

Microarray data analysis. Microarrays were scanned and analyzed as previously described by Anjum et al. (1) with modifications. DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Inc., Union City, CA). Features and the local background intensities were detected and quantified with GenePix 4.0 software (Axon Instruments, Inc.). Poor features were excluded from further analysis if they contained abnormalities or were within regions of high fluorescent background. The data were filtered so that spots with a reference signal lower than the background plus 2 standard deviations of the background were discarded. Signal intensities were corrected by subtracting the local background, and then the Cy5/Cy3 ratios were calculated. To compensate for unequal dye incorporation, data normalization was performed as described previously (1). Briefly, the median natural log (ln) (Cy5/Cy3) for each block was brought to 0 (one block being defined as the group of spots printed by the same pin) with the following equation: $\ln(Ti) = \ln(Cy5i/$

TABLE 1. Bacterial strains used in this study and PCR analysis of CJIE content[a]

RM no.	Strain	Species	Animal source	Country of origin	Penner type(s)	PCR detection of element[b]			
						CJIE1	CJIE2	CJIE3	CJIE4
1045	ATCC 43429	*Campylobacter jejuni*	Human	?	HS:1	−	−	−	−
1046	ATCC 43430	*Campylobacter jejuni*	Calf	?	HS:2	−	−	−	+
1047	ATCC 43431	*Campylobacter jejuni*	Human	?	HS:3	−	−	+	−
1048	ATCC 43432	*Campylobacter jejuni*	Human	Canada	HS:4	−	+	−	−
1050	ATCC 43449	*Campylobacter jejuni*	Human	Canada	HS:23	−	−	+	−
1052	ATCC 43456	*Campylobacter jejuni*	Human	?	HS:36	−	−	−	−
1155	CjT1	*Campylobacter jejuni*	Human	Canada	ND	−	−	−	−
1156	CjT2	*Campylobacter jejuni*	Human	Canada	ND	+	−	+	−
1158	CjT5	*Campylobacter jejuni*	Human	Canada	ND	−	−	−	−
1160	CjT7	*Campylobacter jejuni*	Human	Canada	ND	−	−	−	−
1163	CjT11	*Campylobacter jejuni*	Human	Israel	HS:53	−	−	+	−
1167	CjT28	*Campylobacter jejuni*	Human	Canada	HS:37	−	−	−	−
1170		*Campylobacter jejuni*	Chicken	United States	HS:31	+	−	−	+
1188		*Campylobacter jejuni*	Chicken	United States	HS:2	+	−	+	−
1221		*Campylobacter jejuni*	Chicken	United States	HS:53	+	+	+	+
1244	90A2737	*Campylobacter jejuni*	Human	United States	ND	−	−	−	−
1245	96A5046	*Campylobacter jejuni*	Human	United States	HS:19,38	−	−	−	−
1246	92A3120	*Campylobacter jejuni*	Human	United States	HS:7	+	−	−	−
1247	96A11074	*Campylobacter jejuni*	Human	United States	HS:4,13,64,66	−	−	−	−
1248	96A14504	*Campylobacter jejuni*	Human	United States	HS:4,13,50,64	−	−	−	−
1285		*Campylobacter jejuni*	Chicken	United States	HS:19	−	−	−	−
1409		*Campylobacter jejuni*	Turkey	United States	HS:4,64	−	−	−	−
1413		*Campylobacter jejuni*	Chicken	United States	HS:10	+	−	+	+
1437		*Campylobacter jejuni*	Turkey	United States	HS:11	−	−	+	+
1443		*Campylobacter jejuni*	Chicken	United States	HS:38,63	−	−	−	−
1449		*Campylobacter jejuni*	Chicken	United States	HS:4,13,19,50,65	−	−	+	+
1464		*Campylobacter jejuni*	Chicken	United States	HS:4,5,13,16,19	−	−	+	+
1477	D445	*Campylobacter jejuni*	Human	United States	HS:19,38	+	−	−	−
1478	D226	*Campylobacter jejuni*	Human	United States	HS:2	−	−	−	+
1479	EDL18	*Campylobacter jejuni*	Human	United States	HS:17,23,36	−	−	−	−
1480	D1117	*Campylobacter jejuni*	Human	United States	HS:2	−	−	−	+
1501		*Campylobacter jejuni*	Chicken	United States	ND	−	−	−	−
1503	ATCC 43462	*Campylobacter jejuni*	Human	Canada	HS:43	+	−	+	−
1507	LCDC 17384	*Campylobacter jejuni*	Human	Germany	HS:10	−	−	−	−
1508	LCDC 17385	*Campylobacter jejuni*	Human	Germany	HS:53	−	−	+	−
1510	LCDC 17402	*Campylobacter jejuni*	Human	Japan	HS:19	+	−	−	−
1511	LCDC 17403	*Campylobacter jejuni*	Human	Japan	HS:19	+	−	−	−
1516	ATCC 33560	*Campylobacter jejuni*	Human	United States	HS:23,36	−	−	+	−
1551	ATCC 43433	*Campylobacter jejuni*	Human	?	HS:5	−	−	−	−
1552	ATCC 43434	*Campylobacter jejuni*	Human	Canada	HS:6	−	−	−	−
1553	ATCC 43435	*Campylobacter jejuni*	Human	?	HS:7	−	−	−	−
1554	ATCC 43436	*Campylobacter jejuni*	Human	Canada	HS:8	−	−	−	−
1555	ATCC 43437	*Campylobacter jejuni*	Goat	?	HS:9	−	−	−	−
1556	ATCC 43438	*Campylobacter jejuni*	Human	?	HS:10	−	−	−	−
1844	D135	*Campylobacter jejuni*	Human	Canada	ND	−	−	−	−
1845	D140	*Campylobacter jejuni*	Human	Japan	ND	+	−	−	−
1847	D142	*Campylobacter jejuni*	Sheep	United States	ND	−	−	−	−
1849	D781	*Campylobacter jejuni*	Chicken	United States	ND	+	−	−	−
1850	D983	*Campylobacter jejuni*	Chicken	United States	ND	+	−	+	−
1851	D1038	*Campylobacter jejuni*	Chicken	United States	ND	−	−	−	−
1852	D1420	*Campylobacter jejuni*	Chicken	United States	ND	+	+	+	−
1853	D1713	*Campylobacter jejuni*	Human	United States	ND	−	−	+	+
1854	D1916	*Campylobacter jejuni*	Human	United States	ND	−	−	+	+
1860	L18	*Campylobacter jejuni*	?	?	HS:55	−	−	−	−
1861	L19	*Campylobacter jejuni*	?	?	HS:42,15	−	−	−	−
1862	NCTC 11168	*Campylobacter jejuni*	Human	United Kingdom	HS:2	−	−	−	−
1863	81116	*Campylobacter jejuni*	Human	United Kingdom	HS:6	−	−	−	−
1864	81-176	*Campylobacter jejuni*	Human	United States	HS:23,36	−	−	−	−
1866	D1917	*Campylobacter jejuni*	Chicken	United States	ND	−	−	+	−
1868	D2990	*Campylobacter jejuni*	Human	United States	ND	+	−	+	−
1892	K21	*Campylobacter jejuni*	Chicken	United States	ND	−	+	+	+
2227	72522	*Campylobacter jejuni*	Chicken	United States	HS:15	+	−	−	−
2229	72737	*Campylobacter jejuni*	Chicken	United States	HS:34	−	−	+	+
2232	72927	*Campylobacter jejuni*	Chicken	United States	ND	+	−	+	+
2239	75009	*Campylobacter jejuni*	Chicken	United States	ND	+	−	+	−
2240	75059	*Campylobacter jejuni*	Chicken	United States	ND	−	+	+	−

Continued on following page

TABLE 1—*Continued*

RM no.	Strain	Species	Animal source	Country of origin	Penner type(s)	PCR detection of element[b]			
						CJIE1	CJIE2	CJIE3	CJIE4
2769		*Campylobacter jejuni*	Chicken	United States	ND	+	−	−	−
1505	ATCC 49299	*Campylobacter coli*	?	?	HS:61	−	−	+	−
1531	ATCC 43478	*Campylobacter coli*	Marmoset	?	HS:28	+	−	+	−
1532	ATCC 43482	*Campylobacter coli*	Human	United States	HS:46	+	−	+	−
1533	ATCC 43485	*Campylobacter coli*	Human	United States	HS:49	−	−	−	−
1857	D2611	*Campylobacter coli*	Human	?	ND	−	−	+	−
1858	D2699	*Campylobacter coli*	Human	?	ND	−	−	−	−
1865	D118	*Campylobacter coli*	Human	?	ND	−	−	−	−
1876	ATCC 43473	*Campylobacter coli*	Human	Belgium	HS:14	−	−	+	−
1878	ATCC 43474	*Campylobacter coli*	Human	Canada	HS:20	−	−	+	−
1897	1926	*Campylobacter coli*	Swine	United States	ND	−	−	−	−
2225	72231	*Campylobacter coli*	Chicken	United States	ND	−	−	−	−
2228	72664	*Campylobacter coli*	Chicken	United States	HS:34	−	−	+	−

[a] Strains in boldface type were examined by microarray. ND, Penner type not determined; ?, unknown.
[b] +, element present; −, element absent.

Cy3i) − *c*, where *T* is the centered ratio, *i* is the gene index, *Cy5* and *Cy3* are the Cy5 and Cy3 intensities, respectively, and *c* is the 50th percentile of all Cy5/Cy3 ratios (1). It should be noted that dye-swapping was performed for 6 strains, and no effects due to differences in dye incorporation on downstream analysis were observed. The comparative genomic hybridization (CGH) analysis of either strain NCTC 11168 or RM1221 versus the reference (NCTC 11168 and RM1221 combined) defined ratio cutoffs for the present, divergent, and absent genes. Based on these data, we defined the status of a gene as present when the Cy3/Cy5 (test/reference) intensity ratio was >0.6, as divergent when the Cy3/Cy5 intensity ratio was between 0.6 and 0.3, and absent when the Cy3/Cy5 intensity ratio was <0.3. These values are similar to intensity ratios utilized in other microarray studies (1, 5, 23). The NCTC 11168 and RM1221 strain-specific spots hybridized to only half of the reference DNA (the Cy5-labeled mixture of NCTC 11168 and RM1221 DNA), increasing the Cy3/Cy5 ratio by twofold. The ratios for these spots were therefore divided by 2 before determining the status of the gene. The presence, divergence, and absence status for all genes was converted into trinary scores (present, 2; divergent, 1; absent, 0). The trinary gene scores for each replicate for all strains were analyzed further with GeneSpring microarray analysis software version 7.3 (Agilent Technologies, Redwood City, CA) and subjected to average-linkage hierarchical clustering with the standard correlation and bootstrapping.

RESULTS AND DISCUSSION

Survey of *C. jejuni* strains for RM1221-like genomic elements. Upon comparing the genomes of strains NCTC 11168 and RM1221, the presence of four CJIEs in RM1221 were the most striking differences between these two strains (4, 19). To determine if these CJIEs were common genomic elements in *C. jejuni* or particular to the strain RM1221, we employed a PCR assay to screen for the presence of individual CJIE genes from a collection of 67 *C. jejuni* strains and 12 *Campylobacter coli* strains from several human clinical, veterinary, and food sources. For CJIE1, 2, 3, and 4, we employed primers to genes CJE0269, CJE0544, CJE1094, and CJE1418 (encoding the CMLP1 MuB transposition protein and the CJIE2, 3, and 4 integrase/recombinase proteins), respectively. As shown in Table 1, 55% (37/67) of the *C. jejuni* strains examined were positive for at least one RM1221-like genomic element and 27% (18/67) were positive for two or more of these CJIEs. Interestingly, these elements were also found in another species of *Campylobacter*, *C. coli*. A total of 58% (7/12) of *C. coli* strains were positive for these elements, although they only possessed either CJIE1 or CJIE3. Although a high percentage of the tested strains were found to have these CJIEs, it is possible that this PCR method may be limited, since only a single gene was used to screen for the presence of the CJIEs. Therefore, the analysis was expanded by using a *C. jejuni* whole-genome DNA microarray to simultaneously assess for the presence or absence of several genes that comprise the various CJIEs.

Validation of the custom *C. jejuni* microarrays. A multistrain *C. jejuni* microarray was constructed based on sequence data from the genome strains NCTC 11168 and RM1221 (4, 16). The DNA microarray comprised 1,530 genes from NCTC 11168 and 227 genes from RM1221 (see Materials and Methods). To validate this DNA microarray in specifically distinguishing the genome strains, a reference DNA mixture containing equal amounts of genomic DNA from both NCTC 11168 and RM1221 was labeled with Cy5 and was then competitively hybridized with either Cy3-labeled NCTC 11168 or Cy3-labeled RM1221 genomic DNA. This CGH analysis of either strain NCTC 11168 or RM1221 versus both strains NCTC 11168 and RM1221 combined (reference) defined the ratio cutoffs for the present, divergent, and absent genes. Based on these data, we defined a gene's status as present when the Cy3/Cy5 (test/reference) intensity ratio was >0.6, as divergent when the intensity ratio was between 0.6 and 0.3, and absent when the intensity ratio was <0.3.

Figure 1 depicts the gene status for all RM1221 genes based on the normalized Cy3/Cy5 fluorescence intensity ratio using strain NCTC 11168 as the test strain (Cy3 labeled). These results demonstrated that a gene present in both strains produced a signal intensity ratio of around 1, as visualized in Fig. 1. These results also confirmed that the genomic DNA of either strain RM1221 or NCTC 11168 hybridized equally well to features on the DNA microarray representing genes common to both strains. In contrast, features on the DNA microarray targeting the regions and genes known to be distinct between strains NCTC 11168 and RM1221 were identified by a low-intensity ratio of <0.3, as visualized in Fig. 1. In previous microarray studies (3, 20, 23), most absent or divergent genes clustered in several genomic regions in the NCTC 11168 genome. Specifically, Taboada et al. (23) defined 16 intraspecies hypervariable genomic regions described in Table 2, including the LOS, CAP, and DNA R/M genes. Interestingly, comparing

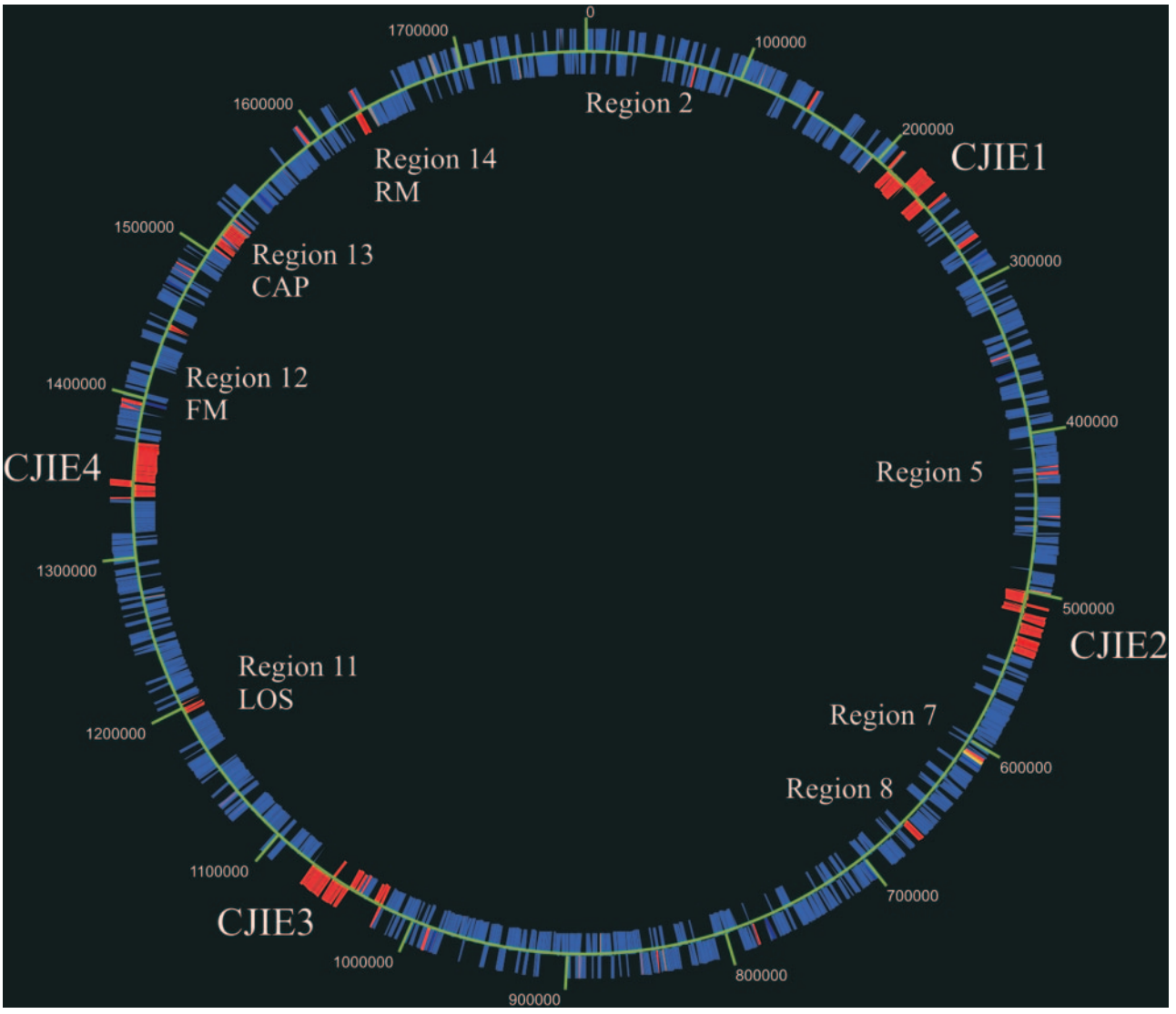


FIG. 1. Genomic index of RM1221 genes in strain NCTC 11168. *C. jejuni* gene status was determined by DNA microarray analysis. The gene status is color coded as follows: blue, present; yellow, variable; red, absent; gray, no data. For cutoffs of absence and presence predictions, refer to Materials and Methods. The genes are represented on the circular RM1221 chromosome. Prominent hypervariable regions are indicated (Table 2), including the four CJIEs.

the genomes of both sequenced strains demonstrated that strain RM1221 possessed genes distinct from strain NCTC 11168 in 11 of these 16 variable regions and was highly divergent from strain NCTC 11168 in 13 of the 16 variable regions. An additional 17th hypervariable region (Cj0258-Cj0263) identified in previous microarray reports (11, 20) and by sequence comparison (4) was also found in our study to be distinct between strains NCTC 11168 and RM1221. As shown in Fig. 1, the results obtained in our DNA microarray analysis correlated well with sequence analysis (4) and verified 14 regions in strain RM1221 that were distinct from strain NCTC 11168, such as the CAP, LOS, and FM regions. Additionally, our DNA microarrays verified other divergent regions containing 20 or more genes with fluorescence intensity ratios of <0.3 that were determined to be absent in strain NCTC 11168. These divergent regions, also shown in Fig. 1, corresponded to the four CJIEs, described previously (4), which are present in strain RM1221.

Determination of divergent genomic regions in *C. jejuni* strains. Of the 67 *C. jejuni* strains that had been PCR screened for the CJIEs, we examined the genomic diversity of 35 strains by CGH analysis using the *C. jejuni* microarray. This subset of strains was selected because they varied with respect to the presence of the CJIEs, as demonstrated by PCR, and represented a diverse group of both human clinical isolates and animal-related isolates. Using the trinary score for absent or highly divergent genes (trinary score of 0) described above, we observed that 21.5% (385 of 1,786) of the genes represented on the microarray were highly divergent or absent in at least one isolate. We also observed that 31.9% (570 of 1,786) were divergent, highly divergent, or absent (trinary score of 1 or 0) in at least one isolate. Recently, Taboada et al. observed 20% divergent,

TABLE 2. Intraspecies hypervariable regions in 35 *C. jejuni* strains

Region	Start–end in RM1221 (NCTC 11168)	Function and/or gene(s)	No. of strains possessing region similar to:		No. of strains divergent from both
			RM1221	NCTC 11168	
1	CJE0031–CJE0035 (Cj0032–Cj0036)[b]	Type IIS restriction/modification	NA[d]	NA	16
2	CJE0051–CJE0055 (Cj0055c–Cj0059c)[a]	Unknown	11	11	13
3	CJE0170–CJE0175 (Cj0177–Cj0182)[b]	Putative iron transport, biopolymer transport, *tonB*, *exbB1*, *exbD1*	NA	NA	14
17	CJE0308–CJE0313 (Cj0258–Cj0263)[c]	*pyrC*, putative zinc transport	5	19	11
4	CJE0340–CJE0355 (Cj0294–Cj0310c)[c]	Pantothenate and biotin biosynthesis pathway Molybdenum ABC transporter	3	14	18
5	CJE0470–CJE0472 (Cj0421c–Cj0425)[a]	Unknown	10	16	9
6	CJE0530–CJE0538 (Cj0480c–Cj0490)[b]	Unknown, *uxaA*	NA	NA	24
7	CJE0666–CJE0673 (Cj0561c–Cj0571)[a]	Unknown	5	14	16
8	CJE0728–CJE0732 (Cj0625–Cj0629)[a]	Hydrogenase, *hypA*, *hypD*, *hypE* Type III restriction/modification (CJE0731–CJE0732)	3	17	15
9	CJE0828–CJE0844 (Cj0727–Cj0755)[b]	Phosphate-regulated genes (Cj0727–Cj0733) (25) Iron uptake (Cj0752–Cj0755), *tonB3*, *cfrA*	NA	NA	11
18	CJE0944–CJE0947 (Cj0857c–Cj0860)[b]	Unknown	NA	NA	22
10	CJE1047–CJE1056 (Cj0967–Cj0975)[a]	Unknown	4	17	14
11	CJE1278–CJE1281 (Cj1135–Cj1145c)[a]	LOS	3	3	29
12	CJE1485–CJE1532 (Cj1293–Cj1343)[a]	FM, O-linked glycosylation locus	1	5	29
13	CJE1601–CJE1622 (Cj1414c–Cj1449c)[a]	CAP	3	3	29
14	CJE1714–CJE1733 (Cj1543c–Cj1563c)[a]	RM, type I restriction/modification and unknown	3	5	27
15	(Cj1677–Cj1679)[a]	Unknown	21	8	6
16	CJE1888–CJE1896 (Cj1717c–Cj1729c)[a]	*leuA*, *leuB*, *leuC*, and unknown	5	2	28

[a] NCTC 11168 and RM1221 are highly divergent.
[b] NCTC 11168 and RM1221 are not distinguished by microarray, only the number of isolates divergent from both are reported.
[c] NCTC 11168 and RM1221 are highly divergent; however, RM1221 genes are absent from the microarray. Isolates with the same presence/absence pattern as RM1221 for these regions were scored as possessing the RM1221 region.
[d] NA, not applicable, since NCTC 11168 and RM1221 are not distinguished by microarray.

highly divergent, or absent in their data set (23). The additional diversity that we observed was likely due to the addition of RM1221 genes on our microarray. Along with the 17th hypervariable region, we identified an 18th intraspecies hypervariable region (Cj0857c-Cj0860) that was similar between strains NCTC 11168 and RM1221. Each of the 18 intraspecies hypervariable genomic regions showed divergent genes in at least one of the 35 strains examined in this study, as summarized in Table 2. Indeed, the hypervariable regions in these strains were often different from regions in both NCTC 11168 and RM1221, especially for regions involved in surface structures (CAP, LOS, and FM) (Table 2). For example, the distinct CAP genes from strain NCTC 11168 (Penner serotype HS:2) and strain RM1221 (Penner serotype HS:53) were present in strains with either Penner serotype HS:2 or HS:53, while divergence in the CAP genes was observed in the isolates with different Penner types (Fig. 2). The recent sequencing of several CAP regions from strains with distinct Penner serotypes has provided greater detail into the nature of the diversity of this region, in particular, distinct genes (10). Similarly, the sequencing of LOS biosynthesis loci has demonstrated the diverse constitution of these regions (6, 18), and our CGH analysis showed that all LOS genes from strains NCTC 11168 or RM1221 were present in the strains that shared the same class of LOS locus (data not shown). Hypervariable region 4 (Table 2), Cj0294c to Cj0310c, in strain NCTC 11168 (CJE0340 to CJE0355 in strain RM1221), containing pantothenate and biotin biosynthesis pathway genes and genes encoding a molybdenum ABC transporter, was distinct between strains NCTC 11168 and RM1221. Since only the genes from the NCTC 11168 region were present on the microarray, it is unclear whether strains that are divergent from strain NCTC 11168 in this region may be more similar to strain RM1221. However, we observed that the hybridization patterns for several isolates were distinct from the pattern observed for strain RM1221, suggesting that additional diversity may be found in this region. Future studies are aimed at further dissecting the role of genomic diversity within this region.

Identification of CJIEs in various *C. jejuni* strains by DNA microarrays. To further assess for the presence of RM1221-like genomic elements in other *C. jejuni* strains, a CGH analysis was conducted by using our *C. jejuni* multistrain DNA microarray. As shown in Fig. 3, the CGH analysis verified the presence of genes of the four CJIEs in most of the strains that were positive in the PCR-based assay. In addition, the DNA microarray analysis demonstrated that diversity was observed within the four CJIEs present in these *C. jejuni* strains relative to strain RM1221 (Fig. 3) and provides evidence for a mosaic or modular arrangement within these integrated elements similar to what has been described for other phage genomes and plasmids (7, 8). A hierarchical clustering was performed to further examine the relationship among each of the CJIEs for

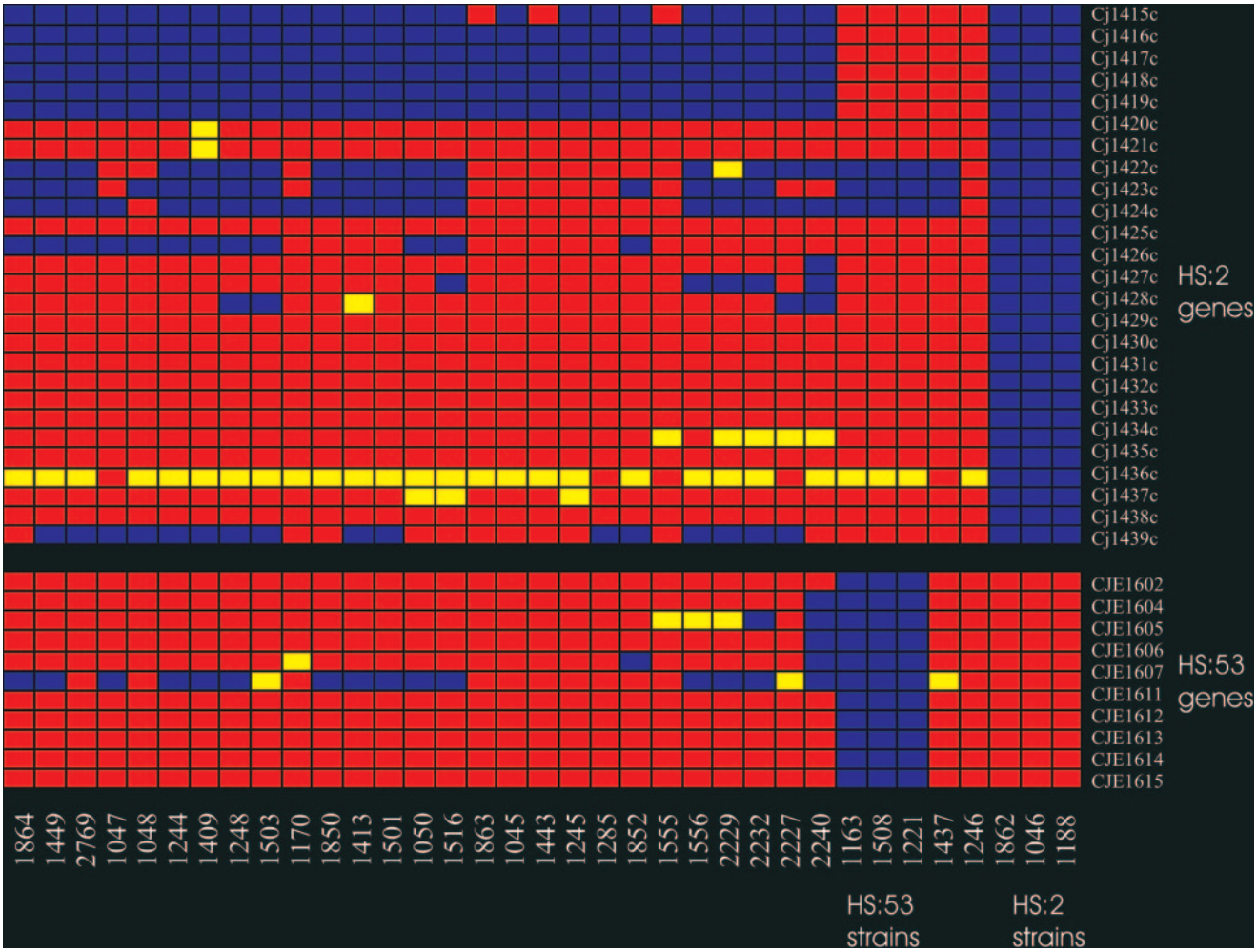
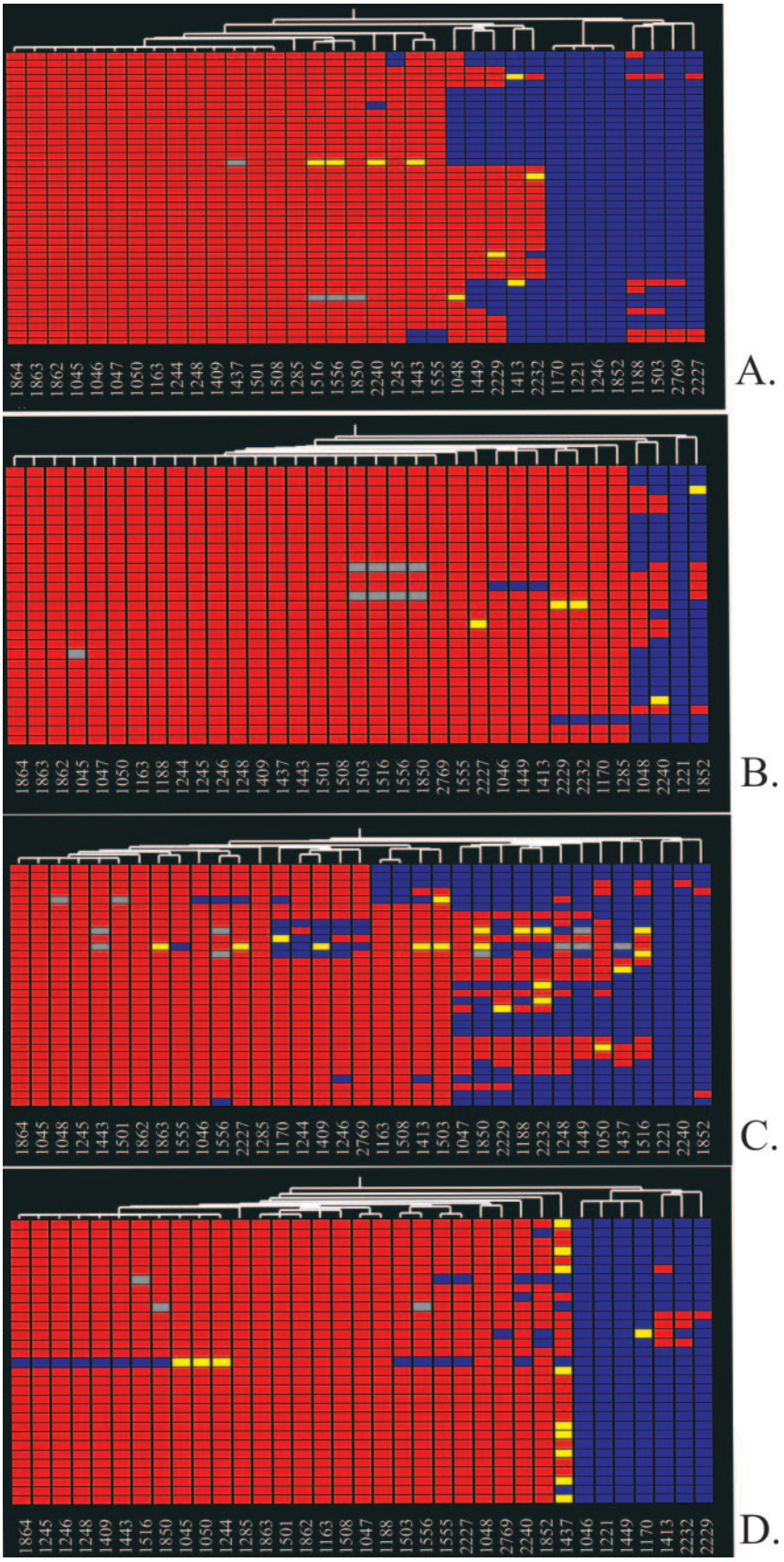


FIG. 2. Detailed genomic index of NCTC 11168 (HS:2) and RM1221 (HS:53) capsular regions in 35 strains of *C. jejuni*. The numbers at the bottom of the figure correspond to the strains in Table 1. The gene status is color coded as follows: blue, present; yellow, variable; red, absent; gray, no data. For cutoffs of absence and presence predictions, refer to Materials and Methods.

each isolate using a standard correlation function (see Materials and Methods) where the linkage distance between strains is represented by branch lengths in the resulting hierarchical cluster. For CJIE1, seven PCR-positive strains formed a cluster with strain RM1221 (distance score = 0.064) (Fig. 3A). Three of these strains (RM1170, RM1246, and RM1852) were identical to strain RM1221 and formed a distinct subcluster (distance score = 0.009) with no divergent or absent genes while those remaining (RM1188, RM1503, RM2769, and RM2227) had many of these genes, were contiguous, and had no more than 8/42 genes absent or divergent (Fig. 3A). Two other PCR-positive strains (RM1413 and RM2232) were more divergent in CJIE1 than RM1221, with at least 15 divergent genes, and these strains clustered (distance score = 0.283) with three CJIE1 PCR-negative strains (RM1048, RM1449, and RM2229) that shared a contiguous set of genes (CJE0227 to CJE0241) encoding putative prophage tail and base plate proteins. These results suggest that such contiguous prophage genes (or modules) have recombined into novel elements distinct from CJIE1 in strain RM1221. The other CJIE1 PCR-negative strains possessed only 2 or fewer of the 42 CJIE1 genes that were represented in the DNA microarray.

Hierarchical clustering of the strains according to CGH results for CJIE2 and CJIE4 genes demonstrated that the PCR-positive strains for each element formed distinct clusters with strain RM1221 (Fig. 3B and D, respectively). All of the CJIE2 PCR-positive strains (RM1048, RM1852, and RM2240) possessed elements with modular composition that were distinct from CJIE2 of RM1221. Interestingly, CJIE2 and CJIE4 possessed the same module of genes, CJE0590 to CJE0598 and CJE1442 to CJE1451, respectively, that were nearly identical at the sequence level (4) and indistinguishable by CGH. We observed that all of the CJIE2 PCR-positive strains (RM1048, RM1852, and RM2240) and all of the CJIE4 PCR-positive strains (RM1046, RM1170, RM1413, RM1449, RM2229, and RM2232) contained this module (data not shown). Therefore, it is possible that these elements share a pool of modules that were obtained by recombination, as previously proposed by Hendrix et al. (7).

The plasmid-like CJIE3 was represented by the most


1864 1863 1862 1045 1046 1047 1050 1163 1244 1248 1409 1437 1501 1508 1285 1516 1556 1850 2240 1245 1443 1555 1048 1449 2229 1413 2232 1170 1221 1246 1852 1188 1503 2769 2227
A.
1864 1863 1862 1045 1047 1050 1163 1188 1244 1245 1246 1248 1409 1437 1443 1501 1508 1503 1516 1556 1850 2769 1555 2227 1046 1449 1413 2229 2232 1170 1285 1048 2240 1221 1852
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D.

PCR-positive strains in our sample set, yet this element appears to be the most diverse region, forming four clusters, (Fig. 3C). As with CJIE1, CJIE2, and CJIE4, the divergent CJIE3 elements in the other strains appeared to have a mosaic arrangement of genes. Particular CJIE3 gene modules were found in both PCR-positive and PCR-negative strains. All 14 PCR-positive strains possessed at least one divergent gene in the CJIE3 element compared to strain RM1221, with two strains, RM2240 and RM1852, forming a subcluster with RM1221 (distance score = 0.053). Four PCR-positive strains, RM1163, RM1508, RM1413, and RM1503, possessed less than 22% (7/33) of the CJIE3 genes present on the array; these CJIE3 genes were mostly contiguous. By CGH, we were able to identify six PCR-negative strains (RM1170, RM1244, RM1409, RM1246, RM2769, and RM1248), each harboring three or more genes from CJIE3. Of particular note was isolate RM1248, which subclustered with seven CJIE3 PCR-positive strains (RM1047, RM1050, RM1188, RM1449, RM1850, RM2229, and RM2232) (distance score = 0.512) and shared 3 gene modules. The CGH analysis for this strain indicated that it possessed gene CJE1094, the gene used in the CJIE3 PCR. The negative result for this strain in our PCR-based assay was probably due to screen sequence differences at the primer binding site(s). Recently, strain RM1047 (TGH 9011) was shown to possess genes that encoded products similar to those encoded by the pathogenicity island of *H. hepaticus*, genes that are absent from strain NCTC 11168 (21). In fact, several of the TGH 9011 genes have DNA sequence homology to the CJIE3 genes of strain RM1221 (Table 3) and were found to be positive based on the CGH analysis. The other five PCR-negative strains (RM1170, RM1244, RM1246, RM1409, and RM2769) possessed at least two genes with sequence similarity to *Campylobacter* plasmids (2, 17) (Table 3). Further analysis would be required to determine if CJIE3 genes in these other strains are chromosomal, as they are in *C. jejuni* strain RM1221, or plasmid borne.

TABLE 3. CJIE3 genes with similarity to *Campylobacter* plasmids or *C. jejuni* strain TGH 9011 contigs

RM1221 gene	Target	No. of identicalnucleotides/ total no. of nucleotides (% identity)
CJE1093	TGH 9011 contig142	324/325 (99)
CJE1094	TGH 9011 contig142	320/324 (98)
	TGH 9011 contig150	308/312 (98)
CJE1100	TGH 9011 contig113	286/288 (98)
CJE1106	TGH 9011 contig091b	335/337 (99)
CJE1112	TGH 9011 contig011	521/533 (97)
CJE1114	TGH 9011 contig078	1,134/1,151 (98)
CJE1122	*C. jejuni* plasmid pTet	181/193 (93)
CJE1126	*C. jejuni* plasmid pTet	254/267 (95)
	C. coli plasmid pCC31	254/267 (95)
	C. jejuni plasmid pCG8245	249/262 (95)
CJE1136	TGH 9011 contig050	112/116 (96)
CJE1138	TGH 9011 contig034a	319/347 (91)
	TGH 9011 contig035	220/232 (94)
CJE1141	TGH 9011 contig021	1,230/1,237 (99)
CJE1153	TGH 9011 contig033	519/523 (99)

CJIE1- and CJIE3-like elements have variable genomic insertion points. Although CGH analysis could demonstrate the presence or absence of the genes for each of the elements, this analysis failed to determine the chromosomal location of the elements in these other *C. jejuni* strains. Aside from the divergent regions, including the RM1221 insertion elements, strains NCTC 11168 and RM1221 display chromosomal synteny elsewhere. If the Mu-like element, CJIE1, of strain RM1221 behaves like other Mu transposable elements, we reasoned that Mu-like elements within other strains of *C. jejuni* might have variable sites of genomic insertion. To determine if CJIE1 in these other strains was located in the same chromosomal position as in strain RM1221, we designed PCR primers to the regions flanking CJIE1. These primers, CJ432 and CJ504, bind to sequences upstream (in CJE0212, bp 206573 to 206596) and downstream (in CJE0274, bp 244371 to 244394) of the CJIE1 element in strain RM1221. Since the two primers were approximately 38 kb apart when a Mu element was present, as in strain RM1221, a standard PCR did not produce an amplicon. Amplification only occurred when the Mu element was absent or located elsewhere in the chromosome. In *C. jejuni* strains NCTC 11168 and 81116, which do not possess CJIE1, amplification resulted in a 400-bp product. As expected, the primers failed to produce an amplicon in strain RM1221 due to the presence of CJIE1. Seventeen other genomic DNAs from strains containing a Mu-like element, RM1156, RM1170, RM1188, RM1246, RM1413, RM1477, RM1503, RM1510, RM1845, RM1849, RM1850, RM1852, RM1868, RM2227, RM2232, RM2239, and RM2759, were amplified with the CJ432/CJ504 primer set. Amplicons were obtained with all 17 tested strains, indicating that the Mu-like element in these strains were in chromosomal locations different from that of strain RM1221. Furthermore, the exact chromosomal location of the Mu-like elements in 4 of these strains, RM1170, RM1246, RM1845, and RM2232, was determined by inverse PCR to be within genes Cj0177, Cj0055c, CJE0140, and CJE1466, respectively. Noteworthy is the presence of the Mu-like phage in strain RM2232 within a CJIE4-like ORF. Based on these observations, Mu-like CJIE1 elements in *C. jejuni*

FIG. 3. Detailed genomic index of the four CJIEs of RM1221 in 35 strains of *Campylobacter jejuni*. The numbers at the bottom of the figure correspond to the strains in Table 1. The gene status is color coded as follows: blue, present; yellow, variable; red, absent; gray, no data. For cutoffs of absence and presence predictions, refer to Materials and Methods. An average linkage hierarchical clustering of the *C. jejuni* strains was compiled in GeneSpring version 7.3 from the CGH data for each element with the standard coefficient correlation and bootstrapping. (A) CJIE1; (B) CJIE2; (C) CJIE3; (D) CJIE4. Two sets of genes, CJE0590 to CJE0598 (CJIE2) and CJE1444 to CJE1451 (CJIE4), show a greater than 95% sequence identity between these elements. Due to this identity, we observed that the CJIE2-PCR positive strains, RM1048, RM1852, and RM2240, cross hybridized with the gene region of CJIE4. Similarly, CJIE4 PCR-positive strains, RM1046, RM1170, RM1413, RM1449, RM2229, and RM2232, cross hybridized with the gene region of CJIE2 (data not shown). We also observed this cross hybridization for CJE0550 and CJE1421, genes encoding putative DNA methyltransferases. Thus, all of these genes were removed from the cluster analysis and are not represented in the figure.

strains appear to insert randomly into locations that are all distinct from the strain RM1221 insertion point. Since Mu phages insert randomly or pseudorandomly into a degenerate 5-bp target sequence, this would then result in a vast number of potential sites for Mu insertion in the chromosome and contribute to the genomic diversity of *C. jejuni*.

The other three elements, CJIE2, CJIE3, and CJIE4, were discovered to be adjacent to tRNA genes. This is commonly observed in other bacterial pathogens, including the *Salmonella* pathogenicity island 1 and the locus of enterocyte effacement in *Escherichia coli* (9). The chromosomal location of CJIE3 was determined for several *C. jejuni* strains that were positive for this element. We designed a PCR primer set, CJ1230-2/CJ1299-2, to the regions flanking CJIE3 in strain RM1221. Strains NCTC 11168 and 81116 both lack CJIE3; therefore, a PCR amplicon was produced with this primer set. As expected, no fragment was amplified by PCR when strain RM1221 was examined due to the presence of the approximately 40-kb CJIE3. Of 23 *C. jejuni* strains examined for the chromosomal location of CJIE3, only 7 yielded an amplicon by PCR, indicating that CJIE3 in each of these 7 strains was inserted at a site different from strain RM1221 or that the CJIE3 genes in these strains were plasmid borne. In contrast, 16 *C. jejuni* strains possessed the element at the same position as strain RM1221, since the DNA of these strains did not amplify with the primer set, CJ1230-2/CJ1299-2. The fact that many of the CJIE3s in the tested strains are in the same chromosomal location as strain RM1221 may be due to the nature of the insertion. If CJIE3 requires insertion into a tRNA site, as in RM1221, then only a limited number of potential integration sites will be available. Determination of the locations of CJIE2 and CJIE4 in different *C. jejuni* strains will require additional analysis.

Concluding remarks. While several CGH studies have demonstrated regions of high variability between strains of *C. jejuni* and strain NCTC 11168 (3, 11, 12, 20, 23), the CGH results reported here, using *C. jejuni* microarrays containing genes from both strain NCTC 11168 and strain RM1221, enabled us to examine additional levels of diversity in *C. jejuni*. Our analysis demonstrated the occurrence of four CJIEs, RM1221-like genomic elements, in other strains of *C. jejuni*. Although a PCR screen also permitted the identification of *C. jejuni* strains possessing CJIEs, results obtained from CGH analysis by DNA microarrays revealed gene diversity between PCR-positive strains within the corresponding CJIEs and also identified several CJIE genes in PCR-negative strains. The presence of bacteriophage-related genes within the CJIEs suggests that various bacteriophage that lysogenize *C. jejuni* contribute to an additional genomic diversity in *C. jejuni*. The modular pattern of CJIE genes in other strains of *C. jejuni* supports the model for bacteriophage genome dynamics, as originally proposed by Hendrix et al. (7), in which a large common bacteriophage gene pool results in the creation of mosaic bacteriophage genomes through horizontal exchange. Indeed, the contribution of bacteriophages and their mosaic structure to the diversification of the *E. coli* and *Salmonella enterica* chromosomes has also been observed by CGH (22, 24). In the present study, we also demonstrated that in all of the *C. jejuni* strains examined, CJIE1, the Mu-like element, was located in different chromosomal locations, possibly due to random insertion events, providing another mechanism to increase the genomic diversity of *C. jejuni*.

Additionally, the global genomic nature of our CGH analysis allowed us to corroborate the clustered nature of highly divergent genes, as observed in previous studies (3, 11, 12, 20, 23). Most highly divergent genes that were identified in our study occurred either within the four CJIEs or within the 17 intraspecies hypervariable regions. Furthermore, with distinct genes from the hypervariable regions of both strains NCTC 11168 and RM1221, the CGH results demonstrated that many of the *C. jejuni* strains in this study were divergent not only from strain NCTC 11168 but also from RM1221. Future work will further explore if these genomic regions could be developed as informative epidemiological tools in distinguishing various strains of *C. jejuni.*

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